Sequential Solid-State and Submerged Cultivation of the White Rot Fungus *Pleurotus ostreatus* on Biomass and the Activity of Lignocellulolytic Enzymes

Qi An, Xue-Jun Wu, Mei-Ling Han, Bao-Kai Cui, Shuang-Hui He, Yu-Cheng Dai, and Jing Si *

Sequential solid-state and submerged cultivation with various lignocellulosic biomasses as a substrate for lignocellulolytic enzyme production by *Pleurotus ostreatus* were assessed by measuring endoglucanase, xylanase, and laccase activities. An unconventional pre-culture method was established by cultivating the *P. ostreatus* mycelia in a solid substrate medium for an initial fungal growth phase, followed by a transition to submerged fermentation through adding a liquid culture medium. The lignocellulolytic enzymes of *P. ostreatus* in different fermentation methods revealed wide differences. The higher yields of endoglucanase (3152 ± 139 U/L), xylanase (3064 ± 40 U/L), and laccase (543 ± 21 U/L) were achieved by using the sequential solid-state submerged method compared to conventional solid-state and submerged cultivation. Generally speaking, sequential solid-state and submerged fermentation of cottonseed hull is favorable for laccase secretion, whereas sequential solid-state and submerged fermentation of corncob provides better production of hydrolytic enzymes. These results revealed that the nature of the lignocellulosic biomass and the fermentation method play an important role in the expression of lignocellulolytic enzymes. This indication would be helpful in optimizing the production of integrated industrial lignocellulolytic enzymes.

Keywords: Pleurotus ostreatus; Lignocellulose biomass; Sequential fermentation; Enzymatic activity; Nutrient medium

*Contact information: Institute of Microbiology, Beijing Forestry University, Beijing 100083, China; * Corresponding author: jingsi1788@126.com*

INTRODUCTION

Replacement of fossil resources with biofuels, eco-friendly resources derived from renewable lignocellulose, has attracted great interest in recent years because of concerns for the ecological environment (Demirbas 2008). Lignocellulosic biomass containing a high proportion of lignocellulose, which has the potential for bioconversion, is particularly abundant and renewable. The white rot fungus *Pleurotus ostreatus* (Jacq.) P. Kumm., which grows on lignocellulose biomass, is one of the most efficient methods for recycling these materials and producing fuels. The use of lignocellulose biomass depends on the capacity of white rot fungus *P. ostreatus* to produce lignocellulolytic enzymes (Buswell *et al.* 1995). Additionally, white rot fungi are known producers of extracellular lignocellulolytic enzymes.

The white rot fungus *Pleurotus ostreatus* produces a wide range of extracellular lignocellulolytic enzymes (Sun *et al.* 2004). The lignocellulolytic enzymes include lignin peroxidase (LiP), manganese peroxidase (MnP), and laccase (Lac), which are involved in

lignin degradation (Gomes *et al.* 2009). Also included are hydrolytic enzymes, cellulases, and hemicellulases (Membrillo *et al.* 2008; Isikhuemhen and Mikiashvilli 2009), which are involved in cellulose and hemicellulose degradation, respectively. These enzymes are able to degrade lignocellulose into low molecular weight compounds (Leatham 1985; Cohen *et al.* 2002). However, there have been few studies on the evaluation of lignocellulolytic enzyme production by this fungus (Baldrian and Gabriel 2003; Bonatti *et al.* 2004; Elisashvili *et al.* 2008a, b). It is important to evaluate the activity of hydrolytic and ligninolytic enzymes.

The cost of enzymatic hydrolysis of lignocelluloses has been a long-standing difficulty (Cardona *et al.* 2010). Biotechnological applications require a large number of low cost enzymes. For this purpose, the selection of appropriate lignocellulose biomass for fungi to grow and enzyme production is one of the important factors in development of efficient biotechnology. Since the last decade, there have been some studies dealing with lignocellulolytic enzymes produced by *P. ostreatus*. They have shown that the production of such enzymes depends strongly on the substrate composition, strain, ion concentration, and conditions of cultivation (Buswell *et al.* 1995; Stajić *et al.* 2006; Elisashvili *et al.* 2008b). Different culture conditions, including the inoculum preparation, can affect enzyme production, and the significant improved in cellulase activity was observed for spores concentration 10^7 spores/mL with medium containing solka floc as inducer (Domingues *et al.* 2000). The influences of ion type (Saparrat *et al.* 2010), type of nutrient medium (Domingues *et al.* 2000), pH, and temperature (Krishna *et al.* 2000; Sohail *et al.* 2009) have all been investigated.

Previous studies have investigated various aspects of lignocellulolytic enzyme production and fermentation methods. Lignocellulolytic enzymes are currently produced by using either solid-state fermentation (SSF) or submerged fermentation (SmF) (Kang *et al.* 2004; Singhania *et al.* 2010; Farinas *et al.* 2011). However, it seems that there have been few reports on lignocellulolytic enzyme production in submerged fermentation after a solid-state pre-culture step in fungi (Cunha *et al.* 2012), and particularly no report in white rot fungi. There also have been no studies showing comparisons of lignocellulolytic enzyme production using various fermentation methods, including sequential solid-state and submerged fermentation, and conventional solid-state fermentation and submerged fermentation. The aim of the present work was to validate the production of lignocellulolytic enzymes of a white rot fungus *P. ostreatus* strain using a solid-state fermentation step followed by a transition to submerged fermentation method of various lignocellulose biomass as the inducer substrate.

EXPERIMENTAL

Materials

Microorganism

A white rot fungal strain, *P. ostreatus* CCEF89, was obtained from the China Center for Mushroom Spawn Standards and Control (CCMSSC) of the Chinese Academy of Agricultural Sciences (Beijing, China). The organism was maintained on 2% (w/v) malt-extract agar (MEA) plates at 4 °C in the Institute of Microbiology, Beijing Forestry University.

Raw materials

Corncob and cottonseed hull were kindly provided by the China Center for Mushroom Spawn Standards and Control. Fresh poplar wood (*Populus beijingensis*) obtained from the Forest Protection Station of Beijing Forestry University (Beijing, China) was chopped into small pieces. All residues were air-dried and then ground. The particles of size between 20- and 60-mesh were prepared for subsequent use.

Nutrient medium

A nutrient medium was used in all fermentation methods, including the pre-culture and during lignocellulolytic enzyme production. Three different nutrient mediums were used in the experiments, named Nmed 1, Nmed 2, and Nmed 3, and the formulations were as follows: Nmed 1, containing $(NH_4)_2SO_4$ 1.4 g/L, KH_2PO_4 2 g/L, $MgSO_4 \cdot 7H_2O$ 0.2 g/L, peptone 5 g/L, yeast extract 2 g/L, and salt solution (5 mg/L $FeSO_4 \cdot 7H_2O$, 1.6 mg/L $MnSO_4 \cdot H_2O$, 1.4 mg/L $ZnSO_4 \cdot 7H_2O$, and 2.0 mg/L $CoCl_2$); Nmed 2, containing glucose 5 g/L, yeast extract 2 g/L, peptone 5 g/L, $(NH_4)_2SO_4$ 1.4 g/L, KH_2PO_4 2 g/L, and $MgSO_4 \cdot 7H_2O$ 0.2 g/L; and Nmed 3, containing yeast extract 2 g/L, peptone 5 g/L, $(NH_4)_2SO_4$ 1.4 g/L, KH_2PO_4 2 g/L, and $MgSO_4 \cdot 7H_2O$ 0.2 g/L.

Methods

Inoculum preparation

Microorganism growth was carried out in 250-mL flasks containing 100 mL of basic medium: glucose 10 g/L, yeast extract 20 g/L, KH_2PO_4 3 g/L, and $MgSO_4$ 0.5 g/L. The medium was then cultured on a rotary shaker at 26 °C with a speed of 150 rpm. After 5 days, mycelial pellets were harvested and homogenized with a laboratory blender for 30 s at 5000 rpm. The resulting suspension was used as the inoculum.

Solid substrate and submerged fermentation

The solid substrate fermentation (SSF) of the poplar wood, corncob, and cottonseed hull was carried out individually at 26 °C in 250-mL flasks containing 5 g of the lignocellulosic substrate moistened with 15 mL of the nutrient medium. Three samples of each type of lignocellulosic substrate were moistened with Nmed 1, Nmed 2, and Nmed 3, respectively (total of 9 treated samples). All 9 flasks containing the lignocellulosic substrates moistened with the medium were autoclaved at 121 °C for 30 min. Three mL of the homogenized mycelium was used to inoculate the autoclaved flasks containing the lignocellulosic substrates moistened with the medium.

After 6, 8, and 10 days of fungal growth in solid substrate fermentation (SSF), the extracellular enzymes were extracted with 50 mM sodium acetate buffer (pH 5.5). First, 100 mL of the extracting solution was added into every Erlenmeyer flask. Extractions were performed at 120 rpm for 5 h at 10 ± 1 °C (Elissetche *et al.* 2007). Culture liquids were obtained by filtering the cultures through a Whatman No. 1 filter paper. The filtrates were then centrifuged (4 °C, 12000 rpm, 15 min), and the supernatants were used for measurement of enzyme activity (Membrillo *et al.* 2008).

Submerged fermentation (SF) of the poplar wood, corncob, and cottonseed hull was carried out using a rotary shaker at 150 rpm and 26 °C in 250-mL flasks containing 100 mL of the nutrient medium. Each kind of lignocellulosic substrate was individually treated with Nmed 1, Nmed 2, and Nmed 3. All 9 flasks containing the lignocellulosic substrates with medium were autoclaved at 121 °C for 30 min. Three mL of the homogenized

mycelium was then used to inoculate the flasks containing the lignocellulosic substrates/medium.

After 6, 8, and 10 days of fungal growth in the submerged fermentation (SF), culture liquids were obtained by filtering the cultures through a Whatman No. 1 filter paper. The filtrates were then centrifuged (4 °C, 12000 rpm, 15 min) and the supernatants were used for measurement of enzyme activity.

Pre-culture for submerged fermentation (SmF)

The pre-culture was initiated just as the solid-state fermentation (SSF) using the lignocellulosic substrates. However, the moisture content was adjusted to 75% (w/w) with the addition of 15 mL of the nutrient medium in a 250-mL Erlenmeyer flask containing 5 g of dry substrate. The mixture was autoclaved at 121 °C for 30 min, and then 3 mL of the homogenized mycelium was used to inoculate the autoclaved flasks under static conditions at 26 °C. After 1 day, 85 mL of the autoclaved medium used in the pre-culture of solid-state fermentation step were added. The cultivation then continued as submerged fermentation (SF) in the flasks under static conditions or on a rotary shaker at 150 rpm and 26 °C.

After 6, 8, and 10 days, culture liquids were obtained by filtering the cultures through a Whatman No. 1 filter paper. The filtrates were then centrifuged (4 °C, 12000 rpm, 15 min) and the supernatants were used for measurement of enzyme activity.

Enzyme assays

Laccase activity was determined by changes in the absorbance at 420 nm with 1 mM 2,2'-azinobis-[3-ethylbenzthiazoline-6-sulfonate] (ABTS) as substrate. The ABTS was dissolved in a 50 mM sodium acetate buffer (pH 4.2). The assay mixture was performed in a 3-mL cuvette with 50 µL of the adequately diluted culture liquid (Woolfenden and Wilson 1982). One unit of enzyme activity was defined as the amount of enzyme required to oxidize 1 µmol of ABTS per minute.

Endoglucanase (CMCase) activity was estimated by the standard method proposed by Ghose (1987) using a solution of 1% (w/v) carboxymethyl cellulose as a substrate, dissolved in a sodium citrate buffer (50 mM, pH 5.0). Xylanase activity was determined by the method described by Bailey *et al.* (1992) using a 1% w/v solution of beechwood xylan as a substrate, which had been previously dissolved in a sodium citrate buffer (50 mM, pH 5.0). One unit of endoglucanase or xylanase activity was defined as the amount of enzyme releasing 1 µmol of reducing sugar per min under the assay conditions.

All experiments were carried out using three replicates. The data is presented in Tables 1 through 4 and corresponds to the mean values with a standard error less than 10%.

RESULTS AND DISCUSSION

Effect of Fermentation Method on Production of Lignocellulolytic Enzymes

Lignocellulolytic enzymes refer to a system of various enzymes whose combined actions cause the degradation of lignocellulose. The white-rot basidiomycetes have a capability of simultaneously producing ligninolytic and hydrolytic enzymes in the fermentation of lignocellulose (Madan *et al*. 1987; Reddy *et al.* 2003; Sun *et al.* 2004; Elissetche *et al*. 2007; Elisashvili *et al.* 2008a,b).

As the most accepted mechanism for the degradation of cellulose, basidiomycetes utilize a set of hydrolytic enzymes typically composed of endoglucanase, exoglucanase or cellobiohydrolase, and *β*-glucosidase (Zhang *et al.* 2006). In the presence of plant polysaccharides, *P. ostreatus* species are able to produce an extensive range of cellulolytic, hemicellulolytic, and pectinolytic families to promote an efficient degradation of the biomass (Riley *et al.* 2014). In this study, in order to simplify the evaluation, only laccase, endoglucanase, and xylanase activities produced by *P. ostreatus* were used to compare the performance of different fermentation methods.

Tables 1 through 4 show the enzymatic activity results for the three different biomasses and the four different fermentation treatments. For the three kinds of lignocellulosic biomass used as the growth substrate in solid-state fermentation, the *P. ostreatus* secreted almost the same comparatively low levels of enzyme activity. The maximum laccase activity was relatively low (77 ± 4.6 U/L) in SSF by using cottonseed hull with Nutrient medium 1 (N-Nmed 1) after 10 d of activity (Table 1). The range of laccase activities varied from 0 U/L to 543 ± 21 U/L among the different fermentation methods.

The range of endoglucanase activities varied from 15 ± 0.6 U/L to 3152 ± 139 U/L among the different fermentation methods, while the range of xylanase activities varied from 37 ± 2.2 U/L to 3064 ± 40 U/L among the different fermentation methods. The higher production of laccase was achieved by submerged fermentation after pre-cultural cultivation (SmF) on a rotary shaker (543 ± 21 U/L, Table 3, Table 5) or under static conditions (445 ± 36 U/L, Table 4, Fig. 1), than by conventional SSF (77 ± 4.6 U/L, Table 1, Fig 3.) and SF (315 ± 31 U/L, Table 2, Fig. 2).

Low enzyme activity can be obtained when a fungus is grown in a stationary liquid culture (Elisashvili *et al.* 2008a,b). However, a bit higher enzyme activity was obtained through the stationary liquid culture after pre-cultural cultivation (stationary-SmF) than that in the conventional submerged fermentation (SF). This could be explained by the laccase activity and endoglucanase and xylanase activities followed later in this article. The productions of endoglucanase and xylanase were 2807 ± 67 U/L (Table 4, Fig. 3) and 1588 ± 86 U/L (Table 4, Fig. 3), respectively, from stationary-SmF, and they were higher than those in SF 1688 ± 33 U/L (Table 2, Fig. 3) and 1557 ± 90 U/L (Table 2, Fig. 3), respectively. Moreover, the endoglucanase and xylanase activities achieved by agitated-SmF were higher than those in the other fermentation methods involved in this study (Figs. 1-3). The highest production of endoglucanase and xylanase were respectively 3152 ± 139 U/L by using cottonseed hull with Nutrient medium 2 after 10 d and 3064 ± 40 U/L by using corncob with Nutrient medium 2 after 10 d of agitated-SmF fermentation (Table 5, Fig. 3).

The maximum endoglucanase and xylanase activities were 563 ± 20 U/L by using corncob with Nutrient medium 2 after 10 d (Table 1, Fig. 3) and 340 ± 22 U/L by using corncob with Nutrient medium 2 after 8 d in SSF (Table 1, Fig. 2). The maximum endoglucanase and xylanase activities were 1688 ± 33 U/L and 1557 ± 90 U/L by using cottonseed hull with Nutrient medium 2 after 10 d in SF (Table 2, Fig. 3). Obviously, the endoglucanase level observed in the agitated-SmF was much greater than that seen for SSF and SF, nearly 5.6-fold and 1.9-fold. Similarly, xylanase activity obtained from agitated-SmF was almost 2-fold and 9-fold higher than those obtained from conventional SF and SSF.

Table 1. Lignocellulolytic Enzyme Activities of *Pleurotus ostreatus* in Solid-State Fermentation (SSF)

Day	Enzyme	M-Nmed1	M-Nmed2	M-Nmed3	Y-Nmed1	Y-Nmed2	Y-Nmed3	N-Nmed1	N-Nmed2	N-Nmed3
6th	Lac	31±1.1	24±0.6	37±2.7	28±1.8	15±1.7	26±1.0	39±1.8	47±0.8	43±1.3
	CMCase	33±2.5	43±3.1	15±0.6	111±7.5	255±17	68±6.4	140±10	126±7.7	75±6.6
	Xyl	156±2.6	37±2.2	47±2.6	209±14	219±14	128±11	218±17	65±3.6	68±6.0
8th	Lac	3.0±0.3	15±0.1	21±1.9	21±1.7	65±4.7	33±2.9	58±4.3	25±0.5	24±1.9
	CMCase	79±7.3	51±2.4	19±1.1	98±8.5	384±10	75±6.7	130±3.6	96±6.5	91±4.5
	Xyl	202±15	142±1.3	111±8.4	209±5.7	340±22	178±2.8	234±2.1	165±14	136±7.6
10th	Lac	17±0.8	8.3±0.5	52±3.8	32±2.6	28±1.4	22±2.0	77±4.6	51±3.6	47±3.8
	CMCase	52±4.3	72±7.1	66±5.7	164±11	563±20	271±12	157±11	119±8.0	111±9.4
	Xyl	120±11	111±7.1	227±5.3	128±5.9	320±16	219±18	143±8.2	138±2.2	238±15

Data are presented as mean ± standard deviation for triplicates and are expressed as U/L. Lac = laccase; CMCase = endoglucanase; Xyl = xylanase. M-Nmed 1 = poplar wood with Nutrient medium 1 (Nmed 1); Y-Nmed 1 = corncob with Nutrient medium 1 (Nmed 1); N-Nmed 1 = cottonseed hull with Nutrient medium 1 (Nmed 1); M-Nmed 2 = poplar wood with Nutrient medium 2 (Nmed 2); Y-Nmed 2 = corncob with Nutrient medium 2 (Nmed 2); N-Nmed 2 = cottonseed hull with Nutrient medium 2 (Nmed 2); M-Nmed 3 = poplar wood with Nutrient medium 3 (Nmed 3); Y-Nmed 3 = corncob with Nutrient medium 3 (Nmed 3); N-Nmed 3 = cottonseed hull with Nutrient medium 3 (Nmed 3)

Table 2. Lignocellulolytic Enzyme Activities of *Pleurotus ostreatus* in Submerged Fermentation (SF)

Day	Enzyme	M-Nmed1	M-Nmed2	M-Nmed3	Y-Nmed1	Y-Nmed2	Y-Nmed3	N-Nmed1	N-Nmed2	N-Nmed3
6th	Lac	0	12±0.3	9±0.4	15±1.5	66±3.8	23±2.3	0	45±2.6	14±1.3
	CMCase	50±3.8	81±4.3	22±1.9	79±3.6	463±41	140±8.6	90±6.7	116±6.6	53±2.4
	Xyl	172±4.8	144±12	155±3.1	208±5.5	413±15	259±9.0	307±15	140±10	261±22
8th	Lac	17±0.4	23±2.3	7.6±0.4	65±4.7	81±8.0	16±1.3	25±0.4	315±31	38±3.5
	CMCase	45±0.3	198±19.7	19±0.7	63±5.1	974±119	93±7.8	73±7	617±58	50±3.1
	Xyl	168±4.3	196±11	90±1.3	278±16	244±16	118±9.9	448±39	369±36	145±14
10th	Lac	81±8.0	24±2.1	10±0.06	93±6.1	83±5.8	15±1.4	54±3.6	14±1.3	3.6±0.3
	CMCase	56±3.4	1249±94	35±3.0	74±0.4	1565±113	84±7.4	70±6.2	1688±33	50±2.2
	Xyl	338±24	1460±111	109±10	660±48	1301±112	100±9.9	351±27	1557±90	121±9.7

Data are presented as mean ± standard deviation for triplicates and are expressed as U/L. Lac = laccase; CMCase = endoglucanase; Xyl = xylanase. M-Nmed 1 = poplar wood with Nutrient medium 1 (Nmed 1); Y-Nmed 1 = corncob with Nutrient medium 1 (Nmed 1); N-Nmed 1 = cottonseed hull with Nutrient medium 1 (Nmed 1); M-Nmed 2 = poplar wood with Nutrient medium 2 (Nmed 2); Y-Nmed 2 = corncob with Nutrient medium 2 (Nmed 2); N-Nmed 2 = cottonseed hull with Nutrient medium 2 (Nmed 2); M-Nmed 3 = poplar wood with Nutrient medium 3 (Nmed 3); Y-Nmed 3 = corncob with Nutrient medium 3 (Nmed 3); N-Nmed 3 = cottonseed hull with Nutrient medium 3 (Nmed 3)

Table 3. Lignocellulolytic Enzyme Activities of *Pleurotus ostreatus* in Agitated Pre-Culture Fermentation (Agitated SmF)

Day	Enzyme	M-Nmed1	M-Nmed2	M-Nmed3	Y-Nmed1	Y-Nmed2	Y-Nmed3	N-Nmed1	N-Nmed2	N-Nmed3
6th	Lac	495±42	50±4.9	39±3.2	192±15	511±18	21±2.0	543±21	7.4±0.7	98±6.9
	CMCase	102±9.0	131±6.9	70±4.2	263±2.4	1336±119	189±5.6	159±3.0	134±8.4	61±5.6
	Xyl	189±4	299±10	260±6.3	1280±120	785±17	580±11	565±13	175±9.2	431±11
8th	Lac	140±5.5	33±2.8	21±1.9	84±8.0	417±40	100±8.4	198±18	319±1.7	103±8.9
	CMCase	54±2.7	772±61	76±5.9	193±12	1847±142	144±12	123±11	1655±105	126±6.5
	Xyl	233±6.6	838±45	276±25	1298±66	862±26	724±47	477±33	832±31	322±8.7
10th	Lac	122±2.6	53±5.3	29±2.2	141±5.7	148±12	86±3.8	184±15	207±13	40±4.0
	CMCase	91±6.0	2497±213	188±4.5	286±24	2891±180	187±11	148±8.1	3152±139	201±11
	Xyl	290±5.7	2423±153	1495±61	255±22.5	3064±40	1720±158	326±9.2	2562±131	1499±21

Data are presented as mean ± standard deviation for triplicates and are expressed as U/L. Lac = laccase; CMCase = endoglucanase; Xyl = xylanase. M-Nmed 1 = poplar wood with Nutrient medium 1 (Nmed 1); Y-Nmed 1 = corncob with Nutrient medium 1 (Nmed 1); N-Nmed 1 = cottonseed hull with Nutrient medium 1 (Nmed 1); M-Nmed 2 = poplar wood with Nutrient medium 2 (Nmed 2); Y-Nmed 2 = corncob with Nutrient medium 2 (Nmed 2); N-Nmed 2 = cottonseed hull with Nutrient medium 2 (Nmed 2); M-Nmed 3 = poplar wood with Nutrient medium 3 (Nmed 3); Y-Nmed 3 = corncob with Nutrient medium 3 (Nmed 3); N-Nmed 3 = cottonseed hull with Nutrient medium 3 (Nmed 3)

Table 4. Lignocellulolytic Enzyme Activities of *Pleurotus ostreatus* in Stationary Pre-Culture Fermentation (Stationary SmF)

Day	Enzyme	M-Nmed1	M-Nmed2	M-Nmed3	Y-Nmed1	Y-Nmed2	Y-Nmed3	N-Nmed1	N-Nmed2	N-Nmed3
6th	Lac	445±36	66±5.9	1.6±0.08	101±9.2	54±4.0	3.5±0.2	180±11	57±3.2	5.3±0.4
	CMCase	40±3.6	61±2.6	21±1.0	57±2.2	384±10	41±3.6	63±5.8	96±6.5	40±2.3
	Xyl	117±7.1	119±9.4	144±2	128±4.4	340±22	161±8.6	157±13	722±47	141±11
8th	Lac	1.6±0.1	260±17	2.4±0.1	7.7±0.7	208±19	19±0.9	6.2±0.3	123±12	9.3±0.7
	CMCase	53±0.2	306±11	29±2.1	63±4.6	2089±163	66±3.8	76±4.9	1330±120	45±3.9
	Xyl	198±1.8	254±4.1	183±8.7	218±14.2	1066±67	201±16	263±13	692±18	217±17
10th	Lac	3.0±0.3	51±0.1	21±1.9	22±1.2	65±5.7	33±2.9	58±4.3	25±0.5	24±2.1
	CMCase	77±7.1	762±64	59±1.1	98±9.5	2807±67	75±7	130±6.6	1437±142	91±4.5
	Xyl	208±15	342±1.3	191±8.1	290±7.7	1588±86	278±18	324±21	765±14	361±21

Data are presented as mean ± standard deviation for triplicates and are expressed as U/L. Lac = laccase; CMCase = endoglucanase; Xyl = xylanase. M-Nmed 1 = poplar wood with Nutrient medium 1 (Nmed 1); Y-Nmed 1 = corncob with Nutrient medium 1 (Nmed 1); N-Nmed 1 = cottonseed hull with Nutrient medium 1 (Nmed 1); M-Nmed 2 = poplar wood with Nutrient medium 2 (Nmed 2); Y-Nmed 2 = corncob with Nutrient medium 2 (Nmed 2); N-Nmed 2 = cottonseed hull with Nutrient medium 2 (Nmed 2); M-Nmed 3 = poplar wood with Nutrient medium 3 (Nmed 3); Y-Nmed 3 = corncob with Nutrient medium 3 (Nmed 3); N-Nmed 3 = cottonseed hull with Nutrient medium 3 (Nmed 3)

The pre-cultural fermentation method produced more enzyme activity within a shorter time than the conventional solid-state or submerged fermentation methods. The production of laccase obtained from agitated Nmed 1-SmF by using corncob as the substrate inducer reached up to 192 ± 15 U/L (Table 3, Fig. 1) on the 6th day. Under the same conditions, the laccase activity in SSF and SF were only 28 ± 1.8 (Table 1, Fig. 1) and 15 ± 1.5 U/L (Table 2, Fig. 1), respectively. The same conclusion was also found for other processing conditions in this study, only except two (cottonseed hull with Nmed 2 and corncob with Nmed 3). The results also showed the consistency on endoglucanase and xylanase.

The unconventional agitated pre-culture fermentation method (agitated-SmF) therefore had a positive influence on lignocellulolytic enzyme production, and similar results have been found for filamentous fungal morphological characteristics in submerged cultivations, which had an effect on cellulase production (Domingues *et al.* 2000; Cunha *et al.* 2012). Hence, this is the first work reporting on basidiomycetes using this pre-culture fermentation method and it has provided promising results.

Effect of Lignocellulosic Substrate on Production of Lignocellulolytic Enzymes

In previous studies, lignocellulose fermentation was usually focused on coffee husks, leaves, wheat straw, sugar cane bagasse, or a combination of various agricultural residues (Moda *et al.* 2005; Elisashvili *et al.* 2008a, b; Membrillo *et al.* 2008), but almost no study has reported using the materials used in the present study. Several features were noted on extracellular enzyme activities produced during fermentation of different plant raw materials used by *P. ostreatus*. Firstly, the corncob is a suitable growth lignocellulosic substrate for the production of hydrolytic enzymes in different fermentation methods. The highest endoglucanase was 563 ± 20 U/L (Table 1, Fig. 3) in Nmed 2-SSF with corncob as the substrate inducer, and it was nearly 8-fold and 4-fold as effective as those of wood and cottonseed hull as inducers, respectively, under the same conditions. Secondly, cottonseed hull was an appropriate growth substrate for the production of laccase in different fermentation methods in most cases. But, interestingly, laccase activities were lower while using cottonseed hull as the lignocellulosic substrate in SF with Nmed 1 than that of poplar wood and corncob. Additionally, in SF with Nmed 1, the endoglucanase and xylanase obtained from cottonseed hull as the lignocellulosic substrate were even a bit higher than those of poplar wood and corncob. This result had been explained by the fact that lignin will have no more effect on cellulose conversion when lignin is degraded to a threshold level, in other words, complete delignification is not necessary (Zhang *et al.* 2006; Zhu *et al.* 2008; Wang *et al.* 2013).

Effect of Nutrient Medium on Production of Lignocellulolytic Enzymes

In recent years, many studies on the effect of nutrient medium on enzyme production have been reported (Elisashvili *et al.* 2008a,b; Membrillo *et al.* 2008; Isikhuemhen and Mikiashvilli 2009). But most studies focus on the substitute of a nitrogen or carbon source, and fewer articles consider the effect of lignocellulosic substrate as the substrate inducer while lacking a simple C or N source. In the present, a simple comparison was done through enzyme yield.

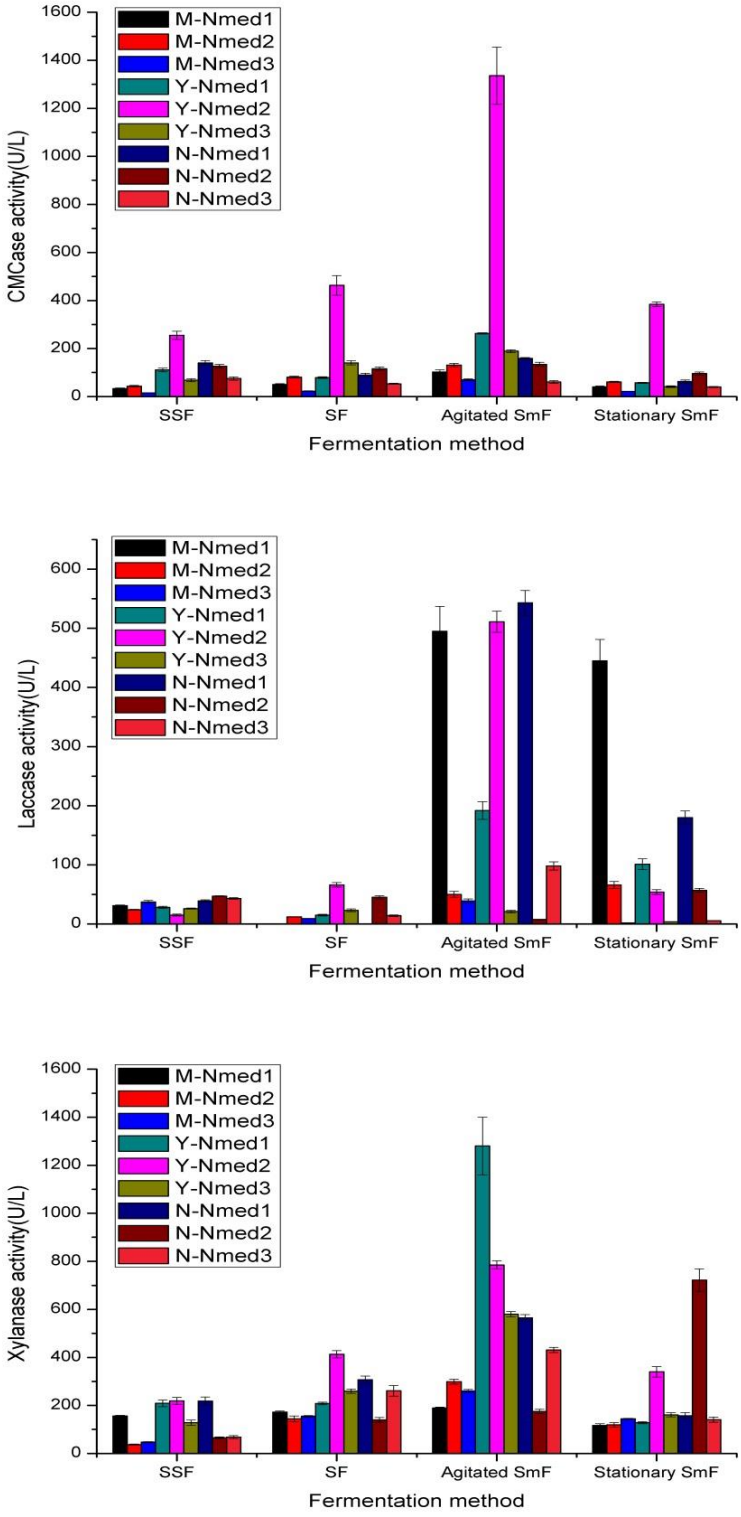

Fig. 1. CMCase activities, laccase activities, and xylanase activities of *Pleurotus ostreatus* grown in different nutrient mediums with various lignocellulosic biomasses as a substrate after 6 d of growth. Average values are reported and were calculated from measurements of three parallel cultures.

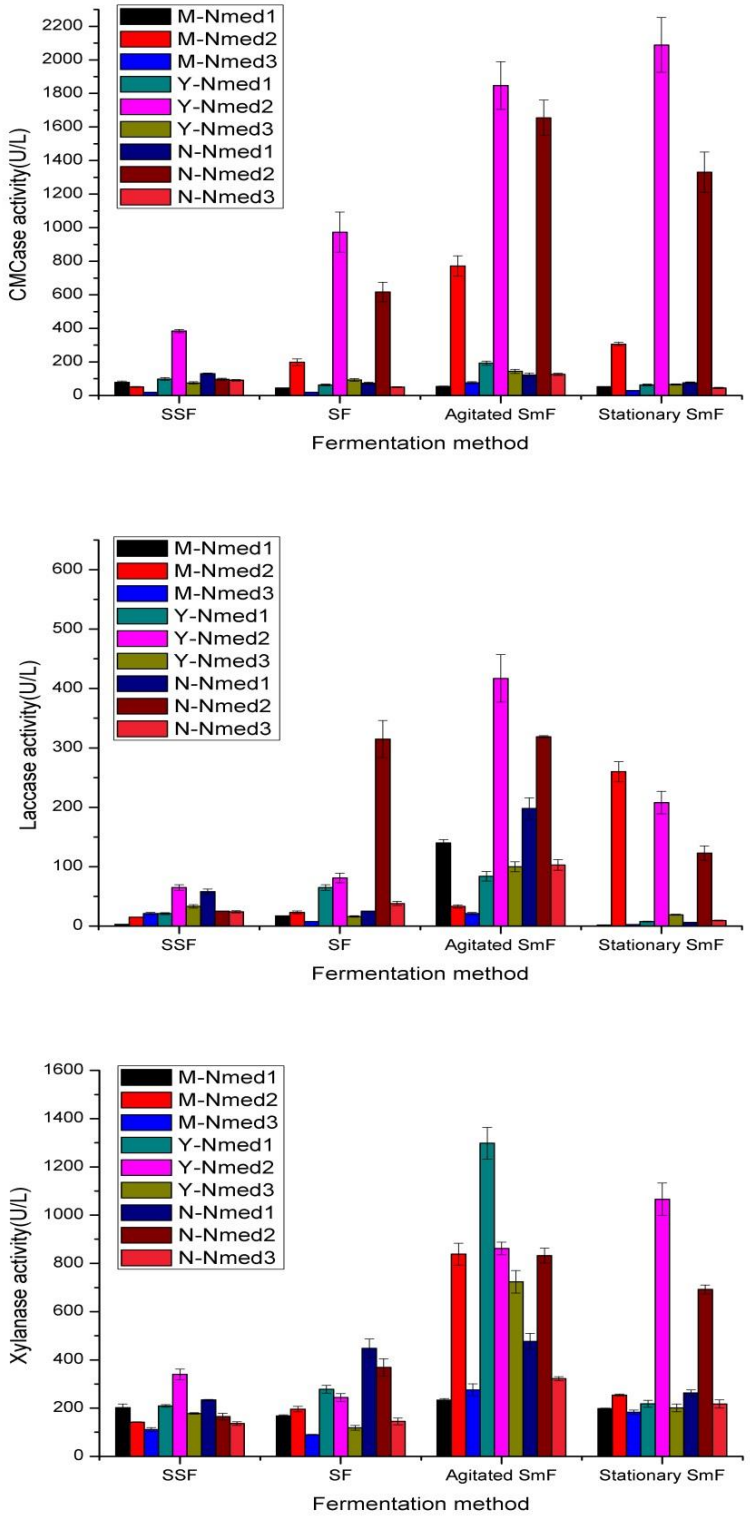

Fig. 2. CMCase activities, laccase activities, and xylanase activities of *Pleurotus ostreatus* grown in different nutrient mediums with various lignocellulosic biomasses as a substrate after 8 d of growth. Average values are reported and were calculated from measurements of three parallel cultures.

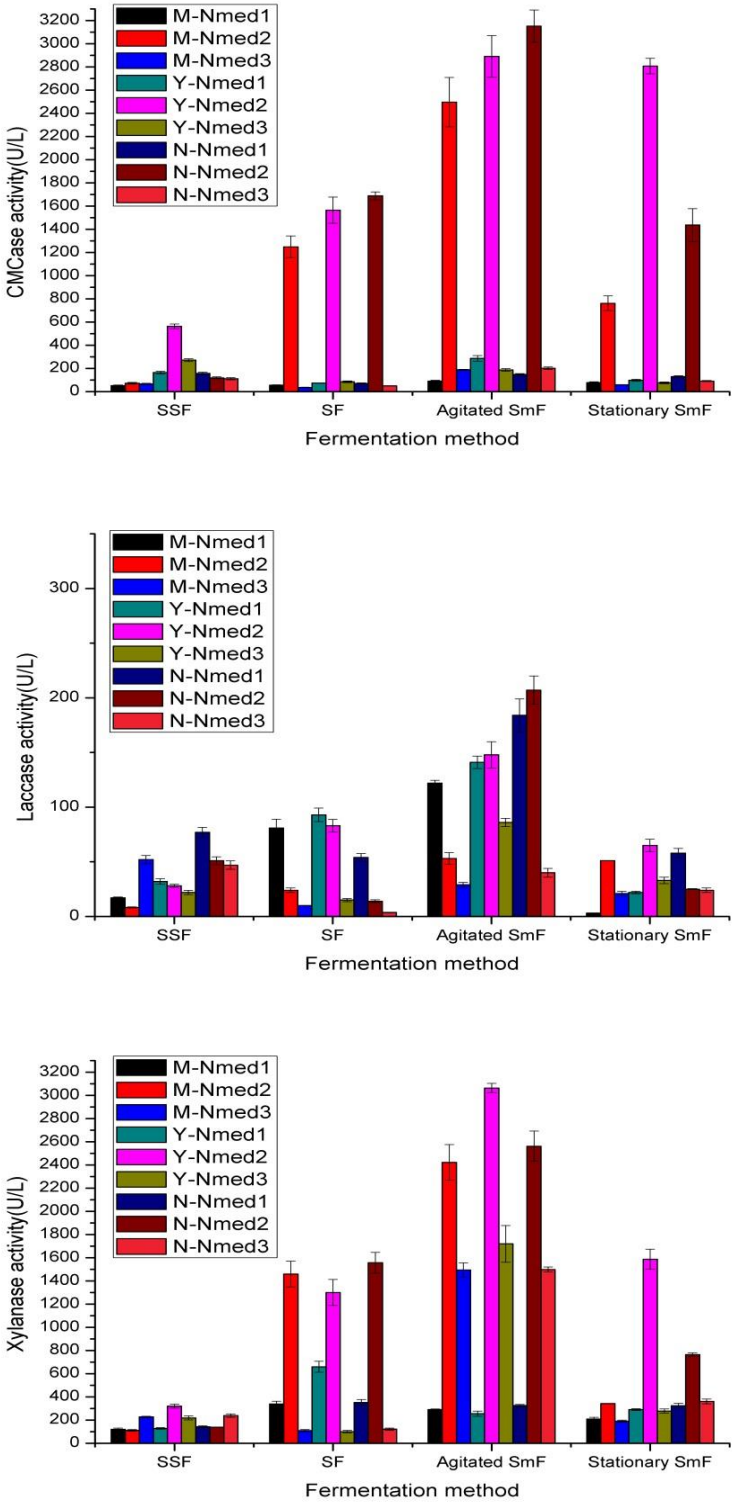

Fig. 3. CMCase activities, laccase activities, and xylanase activities of *Pleurotus ostreatus* grown in different nutrient mediums with various lignocellulosic biomasses as a substrate after 10 d of growth. Average values are reported and were calculated from measurements of three parallel cultures.

Table 5. Maximum Lignocellulolytic Enzyme Activities, Culture Condition, Fermentation Method, and Time of *Pleurotus ostreatus*

Enzyme	Maximum Enzyme Activity (U/L)	Culture Condition	Fermentation Method	Time (d)
Lac	543 ± 21	N-Nmed 1	Agitated-SmF	6
CMCase	3152 ± 139	N-Nmed 2	Agitated-SmF	10
Xyl	3064 ± 40	Y-Nmed 2	Agitated-SmF	10

Data are presented as mean ± standard deviation for triplicates and are expressed as U/L. Lac = laccase; CMCase = endoglucanase; Xyl = xylanase. N-Nmed 1 = cottonseed hull with Nutrient medium 1 (Nmed 1); Y-Nmed 2 = corncob with Nutrient medium 2 (Nmed 2); N-Nmed 2 = cottonseed hull with Nutrient medium 2 (Nmed 2)

The lignocellulolytic enzyme production from Nmed 2 was higher than that from Nmed 1 or 3. The yield of endoglucanase was 3152 ± 139 U/L (Table 3, Fig. 3) in agitated-SmF with Nmed 2 by using cottonseed hull as the substrate, and the xylanase was 3064 ± 40 U/L (Table 3, Fig. 3), which was obtained from agitated-SmF with Nmed 2 by using corncob as the substrate. When the nutrient medium Nemd 1 was used instead of Nmed 2, the minimum endoglucanase was 148 ± 8.1 U/L (Table 3, Fig. 3), a decrease of nearly 22-fold. When the nutrient medium Nemd 1 was used instead of Nmed 2, the minimum xylanase was 255 ± 22.5 U/L (Table 3, Fig. 3), nearly a decrease of 12-fold. One plausible explanation for this phenomenon is that the existence of glucose could have promoted the growth of mycelium. Interestingly, in SF with Nmed 1, the production of laccase activity was 15 ± 1.5 U/L (Table 2, Fig. 1) on the 6th day by using corncob as the substrate, while no laccase activity was detected when poplar wood and cottonseed hull were used as the substrate after 6 d. Also, the laccase activity obtained from Nmed 1 containing salt solution was higher than that from Nmed 2 or Nmed 3 in most cases. However, the production of laccase activity was lower when using corncob as the substrate than when using poplar wood or cottonseed hull as the substrate after the same amount of time in SF and agitated-SmF. The conditions in the agitated pre-culture SmF were particularly obvious. The laccase activity was 543 ± 21 U/L (Table 3, Fig. 1), 495 ± 42 U/L (Table 3, Fig. 1), and 192 ± 15 U/L (Table 3, Fig. 1) with cottonseed hull, poplar wood, and corncob as inducers, respectively.

CONCLUSIONS

1. This study indicated that the biosynthetic potential of basidiomycetes was highly dependent on the method of fungal cultivation, lignocellulosic substrate, and nutrient medium.
2. The combination of solid-substrate and submerged fermentation for lignocellulolytic enzymes was shown to be superior to the conventional solid-state or submerged fermentation methods. Laccase productivity in the agitated-SmF fermentation method was nearly 2-fold and 7-fold higher than that in the submerged and solid-state methods, respectively. Endoglucanase and xylanase activities were similarly higher than that in the submerged and solid-state methods.

3. *Pleurotus ostreatus* growth in the pre-culture fermentation method (agitated-SmF) could get more enzyme activity within a short time.
4. The lignocellulosic substrate and nutrient medium have an effect on enzyme activities. The corncob and cottonseed hull are suitable lignocellulosic substrates for the production of hydrolytic enzymes and laccase, respectively. The laccase activity obtained from Nmed 1 containing a salt solution was higher than that from Nmed 2 or Nmed 3 in most cases.

ACKNOWLEDGMENTS

This research was supported by the National Basic Research Program of China (No. 2014CB138301).

REFERENCES CITED

Bailey, M. J., Biely, P., and Poutanen, K. (1992). "Interlaboratory testing of methods for assay of xylanase activity," *J. Biotechnol.* 23(3), 257-270. DOI: 10.1016/0168-1656(92)90074-J

Baldrian, P., and Gabriel, J. (2003). "Lignocellulose degradation by *Pleurotus ostreatus* in the presence of cadmium," *FEMS Microbiol. Lett.* 220(2), 235-240. DOI: 10.1016/S0378-1097(03)00102-2

Bonatti, M., Karnopp, P., Soares, H. M., and Furlan, S. A. (2004). "Evaluation of *Pleurotus ostreatus* and *Pleurotus sajor-caju* nutritional characteristics when cultivated in different lignocellulosic wastes," *Food Chem.* 88(3), 425-428. DOI: 10.1016/j.foodchem.2004.01.050

Buswell, J. A., Cai, Y. J., and Chang, S. T. (1995). "Effect of nutrient nitrogen and manganese on manganese peroxidase and laccase production by *Lentinula* (*Lentinus*) *edodes*," *FEMS Microbiol. Lett.* 128(1), 81-87. DOI: 10.1111/j.1574-6968.1995.tb07504.x

Cardona, C. A., Quintero, J. A., and Paz, I. C. (2010). "Production of bioethanol from sugarcane bagasse: Status and perspectives," *Bioresour. Technol.* 101(13), 4754-4766. DOI: 10.1016/j.biortech.2009.10.097

Cohen, R., Persky, L., and Hadar, Y. (2002). "Biotechnological applications and potential of wood-degrading mushrooms of the genus *Pleurotus*," *Appl. Microbiol. Biotechnol.* 58(5), 582-594. DOI: 10.1007/s00253-002-0930-y

Cunha, F. M., Esperança, M. N., Zangirolami, T. C., Badino, A. C., and Farinas, C. S. (2012). "Sequential solid-state and submerged cultivation of *Aspergillus niger* on sugarcane bagasse for the production of cellulase," *Bioresour. Technol.* 112, 270-274. DOI: 10.1016/j.biortech.2012.02.082

Demirbas, A. (2008). "Biofuels sources, biofuel policy, biofuel economy and global biofuel projections," *Energy Convers. Manage.* 49(8), 2106-2116. DOI: 10.1016/j.enconman.2008.02.020

Domingues, F. C., Queiroz, J. A., Cabral, J. M. S., and Fonseca, L. P. (2000). "The influence of culture conditions on mycelial structure and cellulase production by

Trichoderma reesei Rut C-30," *Enzyme Microb. Technol.* 26(5), 394-401. DOI: 10.1016/S0141-0229(99)00166-0

Elisashvili, V., Kachlishvili, E., and Penninckx, M. (2008a). "Effect of growth substrate, method of fermentation, and nitrogen source on lignocellulose-degrading enzymes production by white-rot basidiomycetes," *J. Ind. Microbiol. Biotechnol.* 35(11), 1531-1538. DOI: 10.1007/s10295-008-0454-2

Elisashvili, V., Penninckx, M., Kachlishvili, E., Tsiklauri, N., Metreveli, E., Kharziani, T., and Kvesitadze, G. (2008b). "*Lentinus edodes* and *Pleurotus* species lignocellulolytic enzymes activity in submerged and solid-state fermentation of lignocellulosic wastes of different composition," *Bioresour. Technol.* 99(3), 457-462. DOI: 10.1016/j.biortech.2007.01.011

Elissetche, J. P., Ferraz, A., Freer, J., and Rodríguez, J. (2007). "Enzymes produced by *Ganoderma australe* growing on wood and in submerged cultures," *World J. Microbiol. Biotechnol.* 23(3), 429-434. DOI: 10.1007/s11274-006-9243-0

Farinas, C. S., Vitcosque, G. L., Fonseca, R. F., Neto, V. B., and Couri, S. (2011). "Modeling the effects of solid state fermentation operating conditions on endoglucanase production using an instrumented bioreactor," *Ind. Crop. Prod.* 34(1), 1186-1192. DOI: 10.1016/j.indcrop.2011.04.006

Ghose, T. K. (1987). "Measurement of cellulase activities," *Pure Appl. Chem.* 59(2), 257-268. DOI: 10.1351/pac198759020257

Gomes, E., Aguiar, A. P., Carvalho, C. C., Bonfá, M. R. B., da Silva, R., and Boscolo, M. (2009). "Ligninases production by basidiomycetes strains on lignocellulosic agricultural residues and their application in the decolorization of synthetic dyes," *Braz. J. Microbiol.* 40(1), 31-39. DOI: 10.1590/S1517-83822009000100005

Isikhuemhen, O. S., and Mikiashvilli, N. A. (2009). "Lignocellulolytic enzyme activity, substrate utilization, and mushroom yield by *Pleurotus ostreatus* cultivated on substrate containing anaerobic digester solids," *J. Ind. Microbiol. Biotechnol.* 36(11), 1353-1362. DOI: 10.1007/s10295-009-0620-1

Kang, S. W., Park, Y. S., Lee, J. S., Hong, S. I., and Kim, S. W. (2004). "Production of cellulases and hemicellulases by *Aspergillus niger* KK2 from lignocellulosic biomass," *Bioresour. Technol.* 91(2), 153-156. DOI: 10.1016/S0960-8524(03)00172-X

Krishna, S. H., Rao, K. C. S., Babu, J. S., Reddy, D. S., and Krishna, S. H. (2000). "Studies on the production and application of cellulase from *Trichoderma reesei* QM-9414," *Bioproc. Biosyst. Eng.* 22(5), 467-470. DOI: 10.1007/s004490050760

Leatham, G. F. (1985). "Extracellular enzymes produced by the cultivated mushroom *Lentinus edodes* during degradation of a lignocellulosic medium," *Appl. Environ. Microbiol.* 50(4), 859-867.

Madan, M., Vasudevan, P., and Sharma, S. (1987). "Cultivation of *Pleurotus sajur-caju* on different wastes," *Biol. Wastes* 22(4), 241-250. DOI: 10.1016/0269-7483(87)90110-8

Membrillo, I., Sánchez, C., Meneses, M., Favela, E., and Loera, O. (2008). "Effect of substrate particle size and additional nitrogen source on production of lignocellulolytic enzymes by *Pleurotus ostreatus* strains," *Bioresour. Technol.* 99(16), 7842-7847. DOI: 10.1016/j.biortech.2008.01.083

Moda, E. M., Horii, J., and Spoto, M. H. F. (2005). "Edible mushroom *Pleurotus sajor-caju* production on washed and supplemented sugarcane bagasse," *Sci. Agric.* 62(2), 127-132. DOI: 10.1590/S0103-90162005000200006

Reddy, G. V., Babu, P. R., Komaraiah, P., Roy, K. R. R. M., and Kothari, I. L. (2003). "Utilization of banana waste for the production of lignolytic and cellulolytic enzymes by solid substrate fermentation using two *Pleurotus* species (*P. ostreatus* and *P. sajor-caju*)," *Process Biochem.* 38(10), 1457-1462. DOI: 10.1016/S0032-9592(03)00025-6

Riley, R., Salamov, A. A., Brown, D. W., Nagy, L. G., Floudas, D., Held, B. W., Levasseur, A., Lombard, V., Morin, E., Otillar, R., *et al*. (2014). "Extensive sampling of basidiomycete genomes demonstrates inadequacy of the white-rot/brown-rot paradigm for wood decay fungi," *PNAS* 111(27), 9923-9928. DOI: 10.1073/pnas.1400592111

Saparrat, M., Balatti, P. A., Martínez, M. J., and Jurado, M. (2010). "Differential regulation of laccase gene expression in *Coriolopsis rigida* LPSC No. 232," *Fungal Biol.* 114(11), 999-1006. DOI: 10.1016/j.funbio.2010.09.010

Singhania, R. R., Sukumaran, R. K., Patel, A. K., Larroche, C., and Pandey, A. (2010). "Advancement and comparative profiles in the production technologies using solid-state and submerged fermentation for microbial cellulases," *Enzyme Microb. Technol.* 46(7), 541-549. DOI: 10.1016/j.enzmictec.2010.03.010

Sohail, M., Siddiqi, R., Ahmad, A., and Khan, S. A. (2009). "Cellulase production from *Aspergillus niger* MS82: Effect of temperature and pH," *New Biotechnol.* 25(6), 437-441. DOI: 10.1016/j.nbt.2009.02.002

Stajić, M., Persky, L., Friesem, D., Hadar, Y., Wasser, S. P., Nevo, E., and Vukojević, J. (2006). "Effect of different carbon and nitrogen sources on laccase and peroxidases production by selected *Pleurotus* species," *Enzyme Microb. Technol.* 38(1), 65-73. DOI: 10.1016/j.enzmictec.2005.03.026/

Sun, X., Zhang, R., and Zhang, Y. (2004). "Production of lignocellulolytic enzymes by *Trametes gallica* and detection of polysaccharide hydrolase and laccase activities in polyacrylamide gels," *J. Basic Microbiol.* 44(3), 220-231. DOI: 10.1002/jobm.200310376

Wang, W., Yuan, T. Q., Cui, B. K., and Dai, Y. C. (2013). "Investigating lignin and hemicellulose in white rot fungus-pretreated wood that affect enzymatic hydrolysis," *Bioresour. Technol.* 134, 381-385. DOI: 10.1016/j.biortech.2013.02.042

Woolfenden, B. S., and Wilson, R. L. (1982). "Radical cations as reference chromogens in studies of one-electron transfer reactions: Pulse radiolysis studies of 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonate)," *J. Chem. Soc. Perkin Trans. II* 2(7), 805-812.

Zhang, Y. H. P., Himmel, M. E., and Mielenz, J. R. (2006). "Outlook for cellulase improvement: Screening and selection strategies," *Biotechnol. Adv.* 24(5), 452-481. DOI: 10.1016/j.biotechadv.2006.03.003

Zhu, L., O'Dwyer, J. P., Chang, V. S., Granda, C. B., and Holtzapple, M. T. (2008). "Structural features affecting biomass enzymatic digestibility," *Bioresour. Technol.* 99(9), 3817-3828. DOI: 10.1016/j.biortech.2007.07.033

Article submitted: July 1, 2016; Peer review completed: August 7, 2016; Revised version received and accepted: August 18, 2016; Published: August 30, 2016.
DOI: 10.15376/biores.11.4.8791-8805